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1. Introduction

Turmeric (*Curcuma longa* L.), a sacred spice is one of the most important commercial crops known for its aromatic and medicinal properties. It is used as a condiment, dye and cosmetic in addition to its use in religious ceremonies. India is the largest producer, consumer and exporter of turmeric in the world. It is cultivated in South and North Eastern states of India in more than 200 districts spread over 25 states. Export of turmeric in India during 2009-10 was in all time high in terms of value. The export has been 50,750 metric tonnesvalues Rs.351.23 crores. The major production is confined to Andhra Pradesh, Assam, Kerala, Maharashtra, Orissa and Tamil Nadu which accounts for more than 75% of the turmeric produced in the country. It is one of the important spice crops which play a key role in the national economy. The major production is confined to Andhra Pradesh, Assam, Kerala, Maharashtra, Orissa and Tamil Nadu which accounts for more than 75% of the Turmeric produced in the country.

Turmeric is cultivated in India in hot, moist sub-humid to humid and semi arid eco sub regions: lower, middle, upper Brahmaputra plains, Himalayan foot hills, subdued eastern Himalayas, North and South Sahyadris, West coast plains, and in Deccan plateau. Well drained fertile soils ranging from loams, clayey/alluvial, with high organic matter and pH 4.5 -7.5 are suitable for the cultivation of Turmeric.

The crop is vulnerable to a number of fungal diseases of both soil and air borne nature. But bacterial and viral diseases are not so common. The occurrence and severity of diseases depends upon the method of cultivation and the physical factors such as soil moisture that predispose the crop to infection. The important diseases affecting the crop are rhizome rot, leaf spot, leaf blotch, leaf blast, leaf blight, root rot, nematode infestations and storage rots.

Since the etiology of rhizome rot disease in several places appear to be complex, a detailed study on etiology is necessary to identify the real pathogen (s) and also other biotic agents or the secondary intruders that hasten disease development. This requires both conventional and molecular approaches.

Importance of the problem

Rhizome rot caused by various species of *Pythium*, leaf spot caused by species of *Colletotrichum* and leaf blotch caused by *Taphrina maculans* are the important diseases reported in turmeric. Among the diseases rhizome rot is the most destructive one causing economic damage to the crop (Rathiah1982). Crop loss up to a tune of 50% was reported from Telengana region of Andhra Pradesh where the the crop is grown in large scale. An extensive review of literature revealed that the disease is of complex in nature in which various species of fungi and other organisms like nematodes are involved and there exists a controversy in the actual etiology of the disease.

There are several reports on management of the disease through chemical and biological means. However, any management strategies without knowing the actual pathogen (s) involved will not naturally be effective. Though there are reports of using biocontrol agents in managing rhizome rot disease of turmeric, a detailed study is lacking on the source of biocontrol agents used, its potentialities in resisting the pathogen or its adaptability with the environment. Moreover, so far not much work has been done on isolation of indigenous & effective microorganisms antagonistic to the pathogen targeted. Hence a detailed study is warranted to isolate, screen, and characterize effective microorganisms against rhizome rot pathogen of turmeric. It also helps for developing an effective and economical biocontrol strategy for organic system of cultivation. With this background information, the present study is aimed to exercise the details of rhizome rot disease complex in turmeric and also to develop a management strategy to combat the disease using chemical, biological and integrated means. The objectives of the study were

- **To study the intensity and spread of rhizome rot disease in major turmeric growing tracts of South India.**
- **To isolate, investigate pathogenicity and characterize the organism(s).**
- **To isolate, screen, characterize and employ potential antagonistic organisms against rhizome rot disease.**

2. Review of Literature

Crop

The original land of turmeric is still a mystery. There is little evidence that *Curcuma longa* L. is native to India and it seems that it came to India from the ancient region of Cochin China (present day Vietnam) or China through tribal people or Buddhist monks (Ravindran, 2007). Initially it was cultivated as a dye as its bright yellow color used as a coloring agent and later for cosmetic purposes and then as a medicine. Turmeric reached China by 700AD, East Africa by 800AD and West Africa by 1200AD and started becoming popular throughout the world. It is believed to have reached Europe via Silk route from India.

Rhizome rot

Rhizome, the most economic part of turmeric plant is prone to rot disease in almost all turmeric growing regions of India. It is the most destructive of all diseases affecting turmeric and is reported as a major soil borne problem in all turmeric growing tracts. The disease was reported as early as 1934 from Ceylon (Sri Lanka) as caused by *Pythium aphanidermatum* (Park, 1934). In India, the disease was reported from Krishnagiri District of Andhra Pradesh (Ramakrishnan and Sowmini, 1954), Tiruchirappally and Coimbatore in Tamil Nadu, Kasaragod in Kerala (Joshi and Sarma, 1980) and Assam (Rathaiah, 1982a). Shankariah *et al.* (1991) reported high disease incidence from Andhra Pradesh. Crop loss due to the disease varies and crop loss to the tune of 50% was reported from Telengana areas of Andhra Pradesh. The disease occurs during the south west monsoon when the crop is in the active growing stage and waterlogged conditions predispose the plant to infection. Shankariah *et al.* (1991) suggested that there is a positive relationship between continuous rains and rhizome rot incidence. They reported that application of large quantities of tank silt creates drainage problems which will favor disease development.

Etiology

The disease was reported to be caused by different species of *Pythium viz., P. myriotylum, P. graminicolum, P. aphanidermatum.* As early as 1934, the disease was reported to be caused by *P. aphanidermatum* (Park, 1934). Later Ramakrishnan and Sowmini (1954) reported *P. graminicolum* as the causal agent. Sarma *et al.* (1974) recorded brown rot disease in turmeric and isolated *Fusarium* sp. and a nematode, *Pratylenchus* sp. from the affected tissue. Sosamma *et al.* (1979) studied the effect of *Radopholus similis* on turmeric and reported rotting of rhizome and roots and consequent reduction in the yield. Later, Rathaiah (1982a) reported *P. myriotylum* in association with rhizome rot. Ajiri *et al.* (1982) reported the occurrence of maggots of *Mimigrella coerulifrons* in association with the disease affected rhizomes. There are also reports of rhizome rot caused by *Aspergillus*

niger and A. flavus (Sharma and Roy, 1987). The rhizome rot was also reported as caused by *Fusarium solani* from regions like Cuddapah and Guntur Districts of Andhra Pradesh (Dohroo, 1988). Investigations of the causal organisms of turmeric rhizome rot in North Telengana revealed the presence of *Pythium* and *Fusarium solani* (AICRIP on Spices 1996-97). Pathogenic effect of root-knot (*Meloidogyne incognita*) and reniform nematodes (*Rotylenchulus reniformis*) alone and in combination was studied by Haider *et al*. (1996). Though various species of fungi are reported to be associated with rhizome rot of turmeric, species of *Pythium* are found to be the major pathogen. Other species reported are mainly secondary pathogens isolated from both rhizomes and roots in advanced stage of rotting.

There has been a considerable amount of confusion on the etiology of rhizome rot with reports of several fungi and involvement of dipteran maggots at times attributing rhizome rot as a complex involving fungi and insect pests. Premkumar *et al.* (1982) made systematic studies under controlled conditions and concluded that soft rot is primarily caused by *Pythium* species and dipteran maggots play a secondary role of putrefying the rotten tissues. Koya (1990) also reported the role of maggot of *Mimegralla coeruleifrons* as secondary for the causal of rhizome rot in ginger. They also argued that the dipterans do not have the required mouthparts to penetrate the intact tissues of rhizome or roots. They found the association of maggots only with rotten rhizomes and stated that it does not play a significant role in causing the disease. Studies on the rhizome rot complex on turmeric crop in Nizamabad district of Andhra Pradesh during 1979-1988 revealed that *Pythium* sp. is the primary causal organism and the activity of maggots is secondary. However, the rotting incidence is accelerated with the entry of insects into the rhizome (Shankariah *et al.*, 1991).

Careful examination of the disease at various stages proved that the maggots are not the primary causal agents and play a role in the putrefying process. Initial infection by Pythiaceous fungi are soon followed by saprotrophs and often lead to erroneous conclusions. Observations at various stages of infection reveal the secondary role of maggots. If the infection occurs in the early stage of rhizome formation, the entire clump rots. Whereas, infection in the later stage of growth is often restricted to the roots and or secondary/tertiary finger rhizomes. In partially infected rhizomes the presence of maggots and pupa could be seen besides restriction of lesion. Reddy *et al.* (2003) reported *Fusarium solani* as the pathogen of rhizome rot in Andhra Pradesh. All these reports regarding the pathogen(s) that cause rhizome rot in turmeric in southern states of India has made the etiology of rhizome rot a controversial topic for the last three decades.

Biology of the pathogen

Several investigators have studied the dynamics of oospores produced by *Pythium* sp. in

soil, its biology and factors affecting their germination (Peter, 1971; Stanghellini and Burr, 1973a; Davison and McKay, 2003). Oospores of *Pythium* can act as source of primary inoculum at the start of the growing season. Ramakrishnan and Sowmini (1954) studied the biology of the pathogen *P. graminicolum* and showed that the fungus grows over a wide range of pH from 3-9 and the best growth is between pH 7 and 8. Oospores, (perennating structures) production is maximum at pH 6 and 9 and the fungus can tolerate high concentrations of phosphate in culture media while superphosphate @ 125-250 kg ha^{-1} has no effect in reducing the disease incidence and the fungus is sensitive to presence of urea in culture media and soil. Elad and Chet (1987) reported that carbon sources, either provided by synthetic substances or plant root exudates might be involved in the germination of *P. aphanidermatum* oospores. Since the germination and survival of oospores are influenced by several factors such as soil temperature, pH and moisture content, a better understanding of these will provide useful information for the control of many phytopathogenic fungi.

Host range of *Pythium*

Schrandt *et al.* (1994) studied the host range of *Pythium* sp. from carrot and identified six new symptomless hosts in greenhouse pathogenicity tests. They also studied influence of nutrition, temperature, and pH on growth of *Pythium sp.* from carrot. Higginbotham *et al.* (2004) studied the variation in virulence on wheat among isolates of *Pythium* collected from cereal grain fields in eastern Washington. *Pythium* isolates caused a significant reduction in the number of root tips, root length, and length of the first leaf. They reported differences in virulence were detected among species and among isolates within species. Factors that determine pathogen virulence (virulence determinants) should be identified and this information will be useful for the strain selection of biocontrol agents (Tariq and Leonard, 2000).

Diseases of Complex etiology

There are several cases where the etiology of the disease was complex and thereby resulted in crop loss. In ginger, the etiology was complex type since more than one organism was reported in association with rhizome rot. However, Baradwaj *et al.* (1988) reported *P. aphanidermatum* as main pathogen and *F. equiseti* and *F. solani* as seed pathogens. But Chauhan and Patel (1990) reported the involvement of both *Pythium* sp and *Fusarium solani* as causative organisms of rhizome rot disease and confirmed their pathogenicity experimentally. Similarly in cardamom, Radha and Joseph (1974) reported *Phytophthora palmivora* as causative organism of capsule rot. Later Nambiar and Sarma (1974) reported the involvement of *Pythium vexans* in the causal of the disease. Later *Phytophthora nicotianae* was reported by Nair (1979). Sastry (1982) reported *Phytophthora meadii* as pathogen of capsule rot. But a detailed study conducted by

Suseela Bhai (1998) revealed that *Phytophthora media* and *Phytophthora nicotianae var. nicotianae* are two species causing capsule rot in cardamom and also ruled out the role of *Phytophthora palmivora* since it was totally absent during the survey. In cocoyam Agueguia *et al.* (1991) claimed that a complex of three root pathogens comprising *Rhizoctonia solanii, Fusarium solani* and *P. myriotylum* are needed to cause root rot disease. Later Pacumbaba *et al.* (1992), Tambong *et al.* (1999) and Boudjeko (2006) reported that *P. myriotylum* is the unique causal agent of cocoyam root rot disease and others are late colonizers.

Molecular characterization of the pathogen

The ability of polymerase chain reaction (PCR) to amplify specific fragments of DNA has been the basis for many PCR based methods. One of the first uses of PCR in fungal studies was the amplification of ribosomal DNA sequences and the determination of their evolutionary relationships (White *et al.*, 1990). Kageyama *et al.* (1998) characterized isolates of *Pythium ultimum* as well as hyphal swelling groups of *Pythium* on the basis of DNA polymorphisms and their cultural characteristics. The amplified ITS region of rDNA using ITS1 and ITS4 primers followed by RFLP showed no variation between the hyphal swelling group and *Pythium ultimum*. Rouxel *et al.* (1997) developed a molecular criterion to identify the main *Pythium* species involved in cavity spot disease of carrot. They amplified ITS region of rDNA by PCR and digested with restriction enzyme and reported difference in the RFLP pattern of *Pythium* species involved in the disease. Perneel *et al.* (2005) studied the intraspecific variability within 51 isolates of *Pythium myriotylum* from cocoyam (*Xanthosoma sagittifolium*) and other host crops. ITS sequences of isolates *of P. myriotylum* from cocoyam were 99.1–99.7 % identical to sequences deposited in GenBank. They reported molecular evidence that isolates of P. myriotylum that infect cocoyam are distinct from isolates from other crops and have developed a certain degree of host adaptation.

Disease management using biological control agents (BCAs)

The suppression of the disease by biocontrol agents is achieved by the interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment. The use of biological control agents as replacement for chemical fungicides has been addressed in many reports (Harman, 1992; Wafaa, 2002). The use of fungal antagonists to control soil-borne plant pathogens has received much attention in the last three decades. The mycoparasite extends hyphal branches toward the target host, coils around and attaches to it with appressorium-like bodies, and punctures its mycelium (Chet *et al*., 1981; Goldman *et al*., 1994).

Potential of *Trichoderma* as a biological control agent

Considering total fungi used as BCAs in market, almost 50% is shared by *Trichoderma* spp., mostly using as soil/growth enhancers (Whipps and Lumsden, 2001). *Trichoderma* spp. is a fungus that is used as a biocontrol agent against several plant pathogens including *Phytophthora* sp., *Pythium* sp., *Rhizoctonia* sp., and *Fusarium sp.* (Naseby *et al.,* 2000; Sarma and Anandaraj, 1997; Suseela Bhai, 1998; Shalini and Kotasthane, 2007; Anandaraj *et al.,* 1995: Rajan *et al.,* 2002). A number of mechanisms for growth enhancement have been proposed by Harman *et al.* (2004). Tsavkelova *et al.* (2006) reported the production of plant growth stimulators like auxins and cytokinins by *Trichoderma* sp.. Anti microbial activities of *Trichoderma* isolates representing three *Trichoderma* sections were tested by Juan *et al.* (2005). Mycoparasitism by *Trichoderma* spp. primarily involves production of cell-wall degrading enzymes (Howell, 2003). Altomare *et al.* (1999) reported the ability of a *Trichoderma* strain to solubilize soluble or sparingly soluble minerals exhibiting both chelation and reduction. Investigation of Schirmbock *et al.* (1994) on synergism between *Trichoderma* antibiotics and hydrolytic enzymes reported a synergistic effect on *B. cinerea* and *F. oxysporum* hyphal elongation and spore germination using purified antibiotics and enzymes. . Different isolates of the same species of *Trichoderma* can produce different compounds (Luckner, 1990). Culture filtrate of *T. harzianum* was found to suppress white rot in onion (Papavizas *et al.,* 1982). Volatile and non volatile metabolites produced by *Trichoderma* spp. have received attention due to their antibiotic properties. Claydon *et al.* (1987) identified alkyl pyrones from *T. harzianum* that was proved to be highly effective against several fungi under *in vitro* condition. Viterbo *et al*. (2002) have reviewed currently available data on lytic enzymes from the mycoparasitic fungus *Trichoderma* concentrating on the role of its lytic enzymes in the biocontrol of soilborne fungal diseases. Elad *et al.* (1982) reported an increase in the extracellular cellulase activity up to 30-fold by *T. harzianum* when it was grown in *P. aphanidermatum* amended soil in comparison with *R. solani* and *Sclerotium rolfsii* amended soil. Vinale *et al.* (2008) reported that some *Trichoderma* secondary metabolites are directly involved in the *Trichoderma*-plant interactions, and particularly that the compound 6-PAP may be considered to act as an auxin-like compound and/or may act as an auxin inducer.

Induced resistance showed by *Trichoderma* spp.

The use of biological agents to induce the plant defense mechanism against pathogens represents an ecologically friendly alternative to fungicides, which must be repeatedly used for the control of plant diseases (Elad, 2000; El Hassni *et al.,* 2007). Only a few studies so far have dealt with plant defense responses induced by *Trichoderma* (Chang *et al.,* 1997; Yedidia *et al.,* 2000; Martinez *et al.,* 2001). Defense responses by *Trichoderma* during the early stages of roots colonization were demonstrated by de Meyer *et al.* (1998). Induced resistance has been evidenced as one of the mechanisms of *Trichoderma* to

control plant diseases and it represents an ecofriendly alternative to fungicides (Elad, 2000). Recently, Djonovic *et al.* (2006) identified a small protein (Sm1) elicitor secreted by *T. virens*, and demonstrated its involvement in the activation of plant defense mechanisms and the induction of systemic resistance.

Ecology and rhizosphere colonization of *Trichoderma spp.*

Kredics *et al.* (2003) reviewed the influence of environmental parameters *viz.*, temperature, water potential, pH, pesticides, metal ions and antagonistic bacteria on mycoparasitic *Trichoderma* strains on the bioncontrol potential of *Trichoderma* isolates and emphasised the importance of collecting the information about the pesticides or metal compounds. Mondal *et al.* (1996a) reported that acidic pH (5.0) is favourable for the growth of *T. viride* and *T. koningii.* The role of pH in the regulation of extracellular enzyme production was demonstrated by Delgado-Jarana *et al.* (2000) for β-1, 6-glucanase of *T. harzianum.* Enhancement in activity of *Trichoderma* at highly acidic pH has been reported by Harman and Taylor (1988). Jash and Pan (2007) studied antagonistic activity and root colonizing behavior of 10 *Trichoderma* isolates collected from different agro-ecological zone of West Bengal and reported variation in the ability to colonize the rhizosphere soil. Leandro *et al.* (2007) reported the effect of soil manipulation on the population dynamics of indigenous *Trichoderma* spp. on roots and in soil. Their study showed that compost may be used as a substrate to establish and promote survival of *Trichoderma* in strawberry field soil.

Role of *Trichoderma* spp. in reducing pathogen population

Aziz *et al.* (1997) reported the role of root exudates on the rhizosphere colonization by *Trichoderma lignorum* for the control of *Rhizoctonia solani*. They also reported significant reduction in the pathogen population when *T. lignorum* was added as seed coating or conidial suspension. *Trichoderma* spp. have been reported to metabolize the root exudate compounds. Anandaraj and Sarma (1994) have reported the suppression of soil borne inoculum by the incorporation of biocontrol agent in soil during the field evaluation of *T. harzianum* against foot rot disease in black pepper. Prasad *et al.* (2002) suggested that the soil application of *T. harzianum* is an effective method for the control of pigeon pea wilt.

Disease management using chemicals

Thakore *et al.* (1988) evaluated six non systemic and four systemic fungicides against *P. aphanidermatum* causing rhizome rot in ginger. The rhizomes were pre treated with fungicides before planting. Of these, Dithane, M-45, Captafol, Ziride, Captan and Metalaxyl reduced rhizome rot incidence and increased germination and yield. Rathaiah (1987a) reported the control of soft rot of ginger caused by *P. myriotylum* with Ridomil.

Ramachandran *et al.* (1989) studied the effect of Metalaxyl, Fosetyl aluminium, oxadixyl, Etridiazole *in vitro* against *P. aphanidermatum* causing rhizome rot in ginger and reported the efficacy of Metalaxyl to manage the disease. Potassium phosphonate was found effective to control *P. aphanidermatum* and other *Pythium* spp. causing *Pythium* blight of turf grasses. Phillip *et al.* (2009) studied the inhibitory effects of phosphorous acid on *Pythium* spp. *in vitro.* Balakrishnan *et al*. (1996) reported significantly reduced incidence of rhizome rot in ginger caused by *P.aphanidermatum* with captafol, Ridomil-mancozeb, mancozeb and mancozeb + phorate under field conditions.

Management of rhizome rot disease of turmeric using chemicals

According to Rathaiah (1982b), soil drenching with Ridomil at the first appearance of symptoms controlled rhizome rot and increased the yield. Treatment of seed rhizome with Dithane M-45 (0.25%) or Bavistin (1.0%) for 30 minutes prior to storage and at the time of planting and drenching of infected plant beds with Blitox (0.3%) or Bordeaux mixture 1% or copper oxychloride (0.25%) is recommended as a package for controlling rhizome rot and wilt caused by *P. graminicolum*. Rhizome treatment with a combination of mancozeb (0.25%) and quinalphos 0.075% for 15 minutes is the recommended practice (ICAR News letter, 2008). They also recommended the soil drenching with Metalaxyl-mancozeb (0.2%) or mancozeb (0.25%) at 15-20 days interval twice with the first appearance of the symptom for the management of the disease. Joshi and Sharma (1980) suggested the eradication of affected plants by digging out the whole infected plant and drenching the soil with cheshunt compound or ceresan wet (1%) to control the disease. Evaluation of fungicides against *Aspergillus niger* causing rhizome rot of turmeric showed that carbendazim and benomyl are effective even at 100 ppm both under *in vitro* and *in vivo* conditions (Sharma and Roy, 1987). Based on the study on rhizome rot complex in Nizamabad region, Shankariah *et al.* (1991) formulated a package for controlling the disease. The package recommends the use of healthy seed rhizome from disease free area and treating the same before sowing and spraying fungicide *viz.,* carbendazim and drenching the soil with 1% Bordeaux mixture, with the appearance of symptoms.

Sreerama Setty *et al.* (1996) conducted a field trial experiment during 1989-90 and reported Ridomil MZ 72 WP (Metalaxyl) as an effective fungicide against *Pythium* spp. causing rhizome rot of turmeric in Karnataka state, India. Study on rhizome rot of turmeric at Cuddapah region of Andhra Pradesh revealed Ridomil as the most effective fungicide in controlling the disease followed by Mancozeb, Aliette and Thiride (Anandam *et al.*, 1996). Reddy *et al.* (2003) reported the effectiveness of *Trichoderma* along with Ridomil in managing the disease. They also reported the potential of VAM in suppressing rhizome rot caused by *F. solani*.

Integrated disease management (IDM)

The use of multiple antagonists in effective combinations can improve management of diseases using BCA (Lumsden and Papavizas, 1988). IDM using *Trichoderma* spp. and chemicals have been reported by several workers (Sivan and Chet, 1993). Spiegel and Chet (1998) stated that increased biocontrol activity can be achieved by combining two types of BCAs and the combined activity of the antifungal compounds produced by the combination of microorganisms could expand the spectrum of pathogens controlled (Spiegel and Chet, 1998). Harman (2000) reported higher yield in sweet corn in the presence of *Trichoderma* sp. (T 22) than in its absence. The best yields occurred in rows that also received a maneb + metalaxyl (Ridomil) drench to control early-season diseases. With the integrated T-22 + fungicide program, yields were increased 10% relative to the same program without T-22, which was highly significant both economically and statistically. Rajan and Sarma (1997) studied the compatibility of potassium phosphonate (Akomin 40) with different species of *Trichoderma* and *Gliocladium virens* and reported its compatibility for its exploitation for integrated disease management. Thrane *et al.* (2000) reported a non significant difference between the two isolates of *Trichoderma* (*T. harzianum* T3 and *T. atroviride* P1) in their ability to protect cucumber seedlings against *P. ultimum* while the combination of the two fungi resulted in significant difference than each isolate alone. Rakesh and Indra (2007) evaluated fungicides like carbendazim, captan, metalaxyl and MEMC (Methyl ethyl mercuric chloride) against *P. aphanidermatum* causing damping off of tomato. Metalaxyl was found most effective against pathogen at 0.1µg ml^{-1}. Captan and MEMC were effective at 100µgml^{-1}. Application of *T. viride* with metalaxyl resulted in 88 per cent disease control. The combination of metalaxyl + mancozeb was found effective against *Pythium* blight on turfgrass.

3. Materials and Methods

3.1 Study on the intensity and spread of rhizome rot disease in major turmeric growing tracts of South India

3.1.1 Survey for disease incidence in Andhra Pradesh, Kerala, Karnataka and Tamil Nadu

Survey was conducted during 2005 to 2007 in turmeric growing tracts of Andhra Pradesh, Karnataka, Kerala, and Tamil Nadu to study the intensity of rhizome rot disease in turmeric (*Curcuma longa* L). During the survey, a total of 37 different locations in 12 districts of southern states of India were covered.

3.1.2 Collection and isolation of organism(s) from disease specimens and soils from diseased area

3.1.2.1 Isolation of organism(s)

The infected samples were brought to the working place and the infected portions including pseudo stem, roots and rhizomes were used for the isolation. The isolates obtained were maintained in PDA slants for further evaluations.

3.1.2.2 Soil pH estimation

The soil samples collected during survey were analyzed for pH estimation. The soil samples (10 g each) were suspended in 20 ml deionized water and stirred using glass rod and allowed to equilibrate for 30 min. This suspension was used for the estimation of pH using meter.

3.2: Isolation, pathogenicity and characterization of organism (s)

3.2.1 Test for pathogenicity of the isolated organisms (Individual, combined and in sequential inoculations)

The experiment was carried out by inoculating pathogens individually, sequentially and in different combinations to turmeric plants raised in sterile potting mixture. The three suspected pathogens were raised separately in broth culture and inoculated to plants at 3-4 leaf stage (2month old plants), after providing congenial conditions to disease development. The sequential inoculation was done as follows.

T1- *Pythium*	→ *Fusarium*	→*Rhizoctonia*
T2- *Pythium*	→*Rhizoctonia*	→*Fusarium*
T3- *Fusarium*	→*Pythium*	→ *Rhizoctonia*
T4- *Fusarium*	→*Rhizoctonia*	→ *Pythium*
T5- *Rhizoctonia*	→*Pythium*	→ *Fusarium*
T6- *Rhizoctonia*	→*Fusarium*	→ *Pythium*

Similarly combined inoculations were also done as T1- *Pythium* + *Fusarium*, **T2-** *Pythium* + *Rhizoctonia*, **T3-** *Fusarium* + *Rhizoctonia T4- Pythium* + *Fusarium* + *Rhizoctonia*, **T5-** Control **Totally** there were 13 treatments. The observation of disease incidence was critically taken from 3rd day of inoculation.

3.2.2 Characterization of the pathogen (s) using morphological and molecular aids

3.2.2.1. Morphological characterization of the pathogen

A. Colony morphology and growth rate: Morphological characterization of the pathogen was done growing the isolates in various culture media like Potato Dextrose

Agar (PDA), Tomato Agar (TA), Corm Meal Agar (CMA), and Czapek Dox Agar (CZA) at 20-35°C. Phenotypic characters as well as the production of sporangia, oogonia, and antheridia were examined at regular intervals. Sporulation was induced by Grass leaf Method (Robertson 1980) and Tomato Agar Method (L.Y.Guo *etal*.1993). Morphological characters like size of antheridia and oogonia, number of antheridia per oogonia, shape of sporangia, size of oospore, etc. was considered for morphological characterization. Pathogenicity and virulence of isolates were studied using detached leaf inoculation method.

B. sporangial morphology: The sporangial morphology was also studied using slide culture technique. A 3 mm size mycelial plug of 72 h old culture was inoculated at the centre of a slide coated with PDA. The slide was incubated in a humid chamber at 25 ± 1^{o}C for 3 days. The slide was fixed in Lactophenol cotton blue by placing a cover slip over the agar block. This was observed microscopically for the production of sporangia.

C. Virulence of the isolates: The virulence of the isolates was studied using detached leaf method. Young matured leaves of turmeric plants were taken and washed thoroughly in sterile distilled water for three times to remove the debris. Mycelial plugs of 3mm size cut from 72 h old culture of the isolates were placed on the lower surface of the leaves. The mycelial plugs were covered by moistened cotton to prevent the dehydration of the mycelial plug. The leaves were incubated in air tight plastic boxes lined with moistened filter paper. Moistened cotton was also kept inside the box to maintain the humidity inside the box. The boxes were incubated at 25^{o}C for 72 h. Each treatment was replicated thrice. The diameter of the lesions produced after 72 h were recorded.

3.2.2.2 Molecular characterization of *Pythium* isolates: PCR - RFLP Analysis of *Pythium* sp. causing rhizome rot of turmeric

Isolates used: A total of 45 isolates of *Pythium* pathogenic to turmeric were used for the study. DNA of pure culture was extracted. DNA concentrations were estimated visually in ethidium bromide stained agarose gels (0.8%) by comparing band density with known quantity of pBR322 (Genie, Bangalore, India) and also UV spectrophotometry. Template DNA at a concentration of 50ng/µl was used for PCR amplification.

The ITS region of *Pythium* isolates were amplified using the primers ITS-1 (5'-TCCGTAGGTGAAACCTGCGG-3') and ITS-4 (5'- TCCTCCGCTTATTGATATGC-3'). The amplified products were analysed on ethidium bromide stain (2%) along with size standard 500bp ladder (Genei, Bangalore, India), visualized under UV and photographed using alpha imager (Alpha Innotech Corporation).

ITS-RFLP

The amplicons were digested with three restriction enzymes viz. *Taq I*, *Msp I* and *Alu 1*. Data analysis was done by unweighted pair group method using arithmetic averages (UPGMA) analysis of Dice similarity coefficient using the computer program NTSYSpc 2.0.

3.2.3 Study on the biology of the pathogen

3.2.3.1 Oospore production

Oospores production was studied using Grass leaf blade method followed by (Robertson, 1980) and the oospores formed were quantified by Haemocytometer. The viability of the oospores was tested by two methods viz. Plasmolysis method (Jiang and Erwin 1990) and by Tetrazolium bromide (MTT) staining method. (Sutherland *et.al*., 1983).

3.2.3.1. a Quantification of the oospores

The extracted oospores were Oospores were counted in five 20 µl aliquots under the microscope at 10X magnification. The mean of this number was multiplied by the total volume of the suspension to give the total quantity of oospores (Van der Gaag and Frinking, 1997). The oospores thus produced were made into a suspension and adjusted to 1×10^3 ml^{-1} for further studies.

3.2.3.1. b Testing viability of oospores

The oospores extracted were tested for their viability using the method of Sutherland *et al.* (1983) and van Der Gaag (1994).

3.2.3.2. Effect of pH and temperature on the germination of oospores

Oospore germination was studied at different pH ranging from 4.5-8.5 and was incubated at three different temperatures viz. 15, 25 and 35^0C. The percentage of germination was calculated by the number of oospores germinated after 10 days. Oospore germination occurred over a temperature ranging from 15-35^OC. The optimum temperature was found to be 25-35^oC. The optimum pH was found to be 6.0-7.5. The germination occurred at all range of pH but least germination rate was noticed at 4.5 and 5. Some abnormal growth of the germ tube was noted during the germination of the oospore at pH 4.5.

3.2.3.3 Study on the host range of the pathogen

One of the virulent isolates of *P. aphanidermatum* pathogenic to turmeric was tested for its host range studies on six species of Zingiberaceae such *as Curcuma longa, C. zeodaria, C.amada, C.aromatica, Z. officinale* and *C.cassie*. The plants were raised under green house conditions and inoculated with *P. aphanidermatum* keeping *C. longa* as control. Observation on infection was recorded at regular interval.

3.2.3.4 Evaluation of fungicides against *Pythium in vitro*

The fungicide sensitivity of *P. aphanidermatum* was studied using nine fungicides *viz*., Copper oxychloride (50%) WP, Cheshunt compound, Metalaxyl 8%+ mancozeb 64%) WP, Mancozeb (75%) WP, Potassium phosphonate (40%) SC, Carbendazim (12%) + mancozeb (64% WP), Captan (70%) + hexaconazole (5%) WP, Carbendazim (50%) WP and Bordeaux mixture (1%). The fungicide sensitivity of the potential isolates was evaluated by poisoned food technique (Nene and Tapliyal, 1993).

3.3.1 Study on disease management

3.3.1 Isolation of *Trichoderma* from turmeric rhizosphere

The rhizosphere soil samples were collected from major healthy turmeric growing tracts of South India during the survey conducted in 2005-07 seasons and was used for the isolation of *Trichoderma* spp. by dilution plate technique using *Trichoderma* Selective Medium (TSM) (Elad and Chet, 1983).

3.3.2 Evaluation of the antagonistic effect of *Trichoderma* against *Pythium sp.* (*in vitro*)

3.3.2.1 Dual culture assay for preliminary detection of antimicrobial activity

In order to test the antagonistic activities of *Trichoderma* spp. against *P. aphanidermatum, dual* cultures on Potato dextrose agar were established following the method described by Johnson and Curl (1972) was followed. Six Isolates of *Trichoderma* spp. were used. The dual cultures were allowed to grow at $25^{o}C^{\pm}1$for 7 days and the radial growth of the test fungus was recorded after different incubation times. The inhibition of mycelial growth in percent was calculated using the formula described by Edington *et al*. (1971). The growth was also rated by Bell's rating scale.

3.3.2.2 Study on non-volatile metabolites

Culture filtrate of the antagonists was tested for non-volatile effect after incubation of the cultures at $25^{o}C$ for 10 days. The centrifuged and filter sterilized filtrate at a concentration of 10% was added to PDA. The pathogen was inoculated at the centre of the petriplate and was incubated at $25^{o}C$ for five days. The percentage of inhibition was calculated as above.

3.3.2.3 Study on volatile metabolites

The effect of volatile metabolites from *Trichoderma* isolates against *P. aphanidermatum was* assessed according to the method described by Dennis and Webster (1971b). Initially, two Petri plates containing Potato Dextrose agar were individually inoculated with agar disks grown with mycelium of the pathogen and antagonistic isolates. The Petri plate lids were removed, and a plate containing *P. aphanidermatum was* placed over a plate containing *Trichoderma* isolates, then both bottom parts were adjusted and held

together by adhesive tape and incubated at 25 °C for 5 days. The control plates contained *P. aphanidermatum* without the antagonistic isolates. Radial mycelial growth rates of *P. aphanidermatum were* recorded daily and the percentage of inhibition was calculated as mentioned above.

3.3.3 Screening of shortlisted *Trichoderma* isolates for their efficacy to use as biocontrol agent.

Based on *in vitro* inhibitory effect and volatile and non-volatile activities six isolates of *Trichoderma* were shortlisted as potential candidates. Further evaluations were done with this six isolates only.

3.3.3.1 Extracellular enzyme production

Apart from the most common mechanisms of biological control, lytic enzymes produced by *Trichoderma* are likely to play a major role in the biocontrol control. The potential isolates of *Trichoderma* inhibiting *P.aphanidermatum* were studied for their extracellular enzyme production.

(a) Cellulase: Extra cellular cellulase assay was carried out in petri dishes made of CMC agar medium. The culture filtrate were made 1/10 th of its volume by lyophilization and 0.1 ml of the crude lyophilized culture filtrate were added to the wells made by the cork borer. After 48h of incubation at 35^{O}C, the plate was flooded with 0.1% Congo red solution for 15 minutes, and destained using 1M NaCl solution for 10 minutes (Po-Jui Chen, *et al*., 2004). A positive reaction produced a clear zone as halo around the well.

(b) Amylase: Extra cellular amylase production assay was also carried out in petri dishes containing starch agar medium. 0.1 ml of Crude lyophilized culture filtrate were added to the wells in medium and incubated at 35^{O}C for 48 hours. After incubation the plates were poured with Gram's iodine. The clear area around the well showed the production of extracellular amylases (Dubey, *et al*. 2002)

(c) Pectinase: Pectinase production assay was also carried out in petri dishes containing Pectinase Agar medium. 0.1 ml of Crude lyophilized culture filtrate was added to the wells in medium. After 48h incubation at 35^{O}C, the plate was flooded with iodine potassium iodide solution to detect clearance zones. (Marcia Soares *et al.*, 1999)

4.3.2.1.6 Fungicide sensitivity potential *Trichoderma* isolates: The fungicide sensitivity of the potential isolates were studied using 8 fungicides viz. Copper oxychloride, Cheshunt compound, Metalaxyl – mancozeb, Mancozeb, Potassium phosphonate, Carbendazim – mancozeb, Captan – hexoconazole and Carbendazim. Fungicides at different concentrations were incorporated into PDA and inoculated with the isolates. The growth of the isolates was measured as above and calculated the growth inhibition.

3.3.3.2 Induction of plant defense enzymes

Trichoderma species are reported to induce plant defence related enzymes such as Peroxidase (PRX), Polyphenol oxidase (PO), Phenyl ammonia lyase (PAL). These enzymes were studied in turmeric inoculated with the potential isolates of *Trichoderma* found effective in inhibiting the pathogen *invitro.* Leaf, root and rhizome of the turmeric plants inoculated with the potential isolates were macerated in mortar and pestle with 0.1 M sodium phosphate buffer (pH 7.0) at 4^{o}C. the crude extract was centrifuged at 12000 rpm for 20 min. the supernatant taken were used as enzyme extract for assaying PO and PPO enzymes. The tissues were macerated in 0.1M borate buffer (pH 8.8) for PAL enzyme.

Peroxidase assay: 3 ml of 0.05M pyrogallol in 0.1 M sodium phosphate buffer, 100 µl enzyme extract and 100µl hydrogen peroxide were taken as reaction mixture. Changes in absorption were taken at 470nm. Heat killed enzyme extract were used as control.

Polyphenol oxidase assay: the reaction mixture consisted of 1.5 ml of Sodium phosphate buffer, 500µl enzyme extract and 200µl of 0.01M catechol. Changes in absorption were taken at 470nm.

Phenylalanine ammonia lyase assay: The reaction mixture contained 1ml enzyme extract, 3.5 ml borate buffer and 0.1M L-phenyl alanine (pH 8.8). The reaction mixture was incubated at 30^{o}C for 60 min. the reaction was arrested by adding 0.5 ml 1 M Trichloro acetic acid. The absorbance was measured at 290 nm.

3.3.3.3 Fungicide sensitivity of potential *Trichoderma* isolates

The fungicide sensitivity of the potential isolates was evaluated by poisoned food technique (Nene and Tapliyal, 1993) with fungicides *viz*., Captan – hexoconazole, Carbendazim, Carbendazim – mancozeb, Cheshunt compound, Copper oxychloride, Mancozeb, Metalaxyl – Mancozeb and Potassium phosphonate. The different concentrations *viz*., 50, 100, 200, 500, 1000, 2000 and 3000 ppm of each fungicide were incorporated into PDA and poured into 90 mm petridishes at 15ml $plate^{-1}$. Five mm mycelial plugs cut from 72 h old actively growing fungal hyphae were placed at the centre of the petri dish and incubated at 25^{0} C for 96-120 h. Isolates on PDA without incorporating fungicides were kept as control. The radial growth was measured and compared with control.

3.3.3.4 pH tolerance of shortlisted *Trichoderma* isolates

The effects of pH on the growth of the isolates were studied using PDA. The pH of the medium ranging from 4.5-8.5 was adjusted using 0.1 N HCl and 0.1 N NaOH. The medium adjusted to different pH was sterilized by autoclaving at 120^{0}C for 20min. The agar was poured into 90 mm petri plates @ 15ml $plate^{-1}$ and 5 mm mycelial plugs of 72h old culture cut from actively growing fungal hyphae were placed at the centre. The radial growth was measured after 5 days of incubation at 25 ± 1^{0} C and the percent inhibition were calculated

3.3.4 Pot culture evaluation of shortlisted *Trichoderma* isolates against the predominant pathogen

3.3.4.1. Rhizosphere colonization

The potential *Trichoderma* isolates were evaluated under pot culture conditions individually and by challenge inoculation with *P. aphanidermatum*. Individual isolates were tested to understand the effect of the isolates on growth promotion if any as well as rhizosphere colonization, while the combined inoculations were done to understand the effect on disease incidence. The experiment was carried out in a completely randomized block design consisting of thirteen treatments.

3.3.4.2 Evaluation of shortlisted *Trichoderma* isolates against *P. aphanidermatum* by challenge inoculation

For *in vivo* evaluation of promising *Trichoderma* isolates against predominant *Pythium* sp. a pot culture study was conducted in CRD with 7 treatments and 5 replications. The six different *Trichoderma* isolates used were IISR CLT 102, IISR CLT 107, IISR CLT 110, IISR CLT 114, IISR CLT 118 and IISR CLT 121.

3.3.4.2 a Disease Potential Index (DPI) estimation

The rhizosphere soil samples from the respective treatments were collected separately and were used for the detection of pathogen and Disease Potential Index (DPI) by soil dilution end point method (Anandaraj and Sarma, 1990).

3. 3. 5 Disease management trial with chemicals

Four field trials were conducted in two seasons during 2007-2010 at Settiputhur in Coimbatore District in Tamil Nadu, where the disease was reported to be a serious threat to turmeric farmers. Ridges and furrow method of cultivation with irrigation is the traditional method of cultivation practiced in these areas. The trial consisted of 10 fungicide treatments *viz*., Copper oxychloride (0.25%) Cheshunt compound (0.3%) Bordeaux Mixture (1%), Metalaxyl-mancozeb (0.125%), Mancozeb (0.3%), Potassium phosphonate (0.3%), Carbendazim-mancozeb (0.3%), Captan- hexoconazole (0.3%) and Carbendazim (0.5%).Control plot was maintained with out any treatments. The trial was laid out in RBD with six replications.

3.3.5.1 a. Seed dressing, drenching and germination: The rhizomes were treated with fungicides before planting and germination percentage was recorded 60 d after planting.

3.3.5.1. b Estimation of total fungal and bacterial population : The soil samples from each treatment were collected at 30 days intervals after planting up to 180 days. The population of the fungi and bacteria were estimated using soil dilution plating

3.3.5.1. c Disease incidence and yield: The field was monitored regularly for the occurrence of rhizome rot disease among different treatments as well as control. The no. of infected plants showing rhizome rot symptoms were recorded and used to calculate the percentage disease incidence. This was calculated by the no. of plants infected to the total no. of plants in that block. The harvested rhizome from each block was weighed separately and recorded.

3. 6 Disease management trials with shortlisted *Trichoderma* isolates

The isolates of *Trichoderma* short listed as effective from pot culture studies were evaluated in three agroclimatically locations including two disease prone areas and one apparently healthy area. The field trial was conducted in two seasons during the 2007-2009 at different locations which are different for the turmeric cultivation. Traditional cultural practices that have followed by the farmers were followed as such in the respective locations. In Tamil Nadu the trial was conducted in Settiputhur, Coimbatore Dist., and Tamil Nadu where the farmers follow ridges and furrows type cultivation.

3.3.6.1 a. Seed dressing, drenching and germination count: Healthy finger and split mother rhizomes of about uniform size and weight were selected and dipped in spore suspension (cfu $1X10^{8/}ml^{)}$ in water with 5% starch for half an hour @ 1 kg rhizome/lit. The dipped rhizomes were allowed to dry for half an hour in shade and the remaining spore suspension was drenched to the rhizomes of the respective treatments just before mulching. The drenching was done @ 2 lit/bed in raised bed system and @ 10 lit/block in ridges and furrows for each treatment. The soil treatment repeated at 30, 60 and 90 days interval after planting. The disease incidence was recorded at regular intervals.

3. 3. 6. 1. b Estimation of rhizoshere colonization of *Trichoderma* isolates The soil samples were collected at regular intervals. The population of *Trichoderma* was determined by serial dilution technique (Johnson and Curl, 1972) using *Trichoderma* selective medium (Elad and Chet, 1983).

3.3.6.1c Estimation of total fungal and bacterial population

The total fungal and bacterial population present in the soil of different treatment was estimated by the method followed in 3.3.5.1b

3.3.6.1 d Disease incidence and yield

The disease incidence and yield was calculated and recorded by the method followed in 3.3.5.1.c.

3.3.7 Integrated disease management

Field trial for integrated disease management was conducted during 2009-2010 in turmeric fields at Settiputhur. In this trial, fungicide along with biocontrol agents was used. The trial consisted of 7 treatments *viz*., Metalaxyl 8% + mancozeb 64% WP and CLT 107,

Metalaxyl 8% + mancozeb 64% WP and CLT 110 Metalaxyl 8% + mancozeb 64% WP and CLT 107 + CLT 110, Copper oxychloride 50% WP and CLT 107, Copper oxychloride 50% WP and CLT 110, Copper oxychloride 50% WP and CLT 107 + CLT 107, CLT 107+ CLT 110 and control.

3.3.7.1 Seed dressing, drenching and germination count

Healthy finger and split mother rhizomes of about uniform size and weight were selected and dipped in fungicides at 1 kg rhizome L^{-1} of spore suspension. The dipped rhizomes were allowed to dry for 30 min in shade. The chemicals were drenched immediately after planting at 5 L $block^{-1}$. The spore suspensions of potential biocontrol agents containing a CFU of 1 $x10^8$ ml^{-1} were drenched at 10 L $block^{-1}$ in ridges and furrows 30 DAP. The drenching was continued at 60^{th} and 90^{th} DAP. In bed system, the chemicals were drenched at 2 L bed^{-1}. The spore suspensions of biocontrol agents containing cfu of 1 x 10^8 bioagents were drenched 2 L bed^{-1}. The soil treatment using biocontrol agents were repeated at 60^{th} and 90^{th} DAP.

3. 3. 7. 2. Estimation of rhizoshere colonization of *Trichoderma* isolates

The population level of *Trichoderma* isolates in different treatments was estimated by the method followed in 3.3.6.2

3.3.7.3 Estimation of total fungal and bacterial population

The total fungal and bacterial population present in the soil of different treatment was estimated by the method followed in 3.3.5.1.b

3.4 Statistical analysis: Disease incidence and yield data were analyzed using MSTATC (Version 1.41, Russell D. Freed, MSTAT Director and Scott P. Eisensmith, Deputy Director, Crop and Soil Science Department, Michigan State University). For disease incidence, the percentage data were transformed by square root. All other analysis were done using appropriate statistical package are mentioned then and there in the results.

4. Results

4.1 Study on the intensity and spread of rhizome rot disease in major turmeric growing tracts of South India.

4.1.1 Survey for disease incidence in Andhra Pradesh, Kerala, Karnataka and Tamil Nadu.

A total of 37 different locations in 12 districts of southern states of India were surveyed to study the intensity and spread of infection. Symptoms were studied in detail and per cent disease incidence was calculated. Disease incidence varied considerably among different locations. The average disease incidence ranged from 0-32%. Information on mode of

storage of rhizomes, seed treatment, planting date, mulching materials, fertilizer doze, fungicide application, crop rotation and inter cropping practices being adopted during cultivation of turmeric were recorded during the survey. The highest disease incidence (32%) was recorded from Vallabhapuram in Guntur dist. of Andhra Pradesh whereas Cheraooru in A.P and Vellarikunnu in Kerala recorded the least (0%) incidence.

4.1.2 Collection and isolation of organism(s) from disease specimens and soils from diseased area

A total of 118 samples collected from different locations of South India were used for isolation of fungi. *Pythium* spp., *Fusarium* spp., and *Rhizoctonia* spp. were the major organisms isolated from the infected samples. Among the organisms isolated, *Pythium* spp. dominated in majority of the diseased samples followed by *Rhizoctonia spp.* and *Fusarium* spp. Out of 118 samples, 88 samples (74.5%) yielded *Pythium* spp. 38 samples (32.2%) yielded *Rhizoctonia* spp. and 34 samples (28.8%) yielded *Fusarium*. However, there were some samples which yielded a combination of these three organisms Out of total 118 samples, 47 samples (39.83%) showed *Pythium* spp. alone, seven samples (5.93%) *Rhizoctonia* alone and nine samples (7.62%) *Fusarium* alone. Both *Pythium* and *Fusarium* were isolated from 18 samples **(**15.25%) *Pythium* and *Rhizoctonia* from 14 samples (11.86%) and *Fusarium* and *Rhizoctonia* from10samples 8.47%).

4.1.2.2 Study on soil pH: The maximum disease incidence was noticed at Vallabhapuram, Guntur District of Andhra Pradesh where the pH was found to be between 6.27 – 6.81. The other three locations of the same district recorded high disease incidence and all showed pH range 6.00 – 7.50. In Kerala, where the soil pH found to be at the range of 4.20 – 6.1 low DI was noted. The pH of the soil showed a positive correlation with disease incidence (0.525 at 0.01 level of significance).

4.2. Pathogenicity and characterization of organism(s)

4.2.1 Test for pathogenicity of the isolated organisms (Individual, combined and in sequential inoculations)

On individual inoculation studies with *Pythium, Fusarium* and *Rhizoctonia sp.* on turmeric, *P. aphanidermatum* showed infection with in 10 days after inoculation where as the *Fusarium oxysporum* sp. *Rhizoctonia solani* showed no symptoms even after 90 days of inoculation. On sequential inoculation studies, all the treatments showed infection only after inoculation with *Pythium*. The aggravation of the disease was noted in the treatment with *Pythium* inoculated first followed by inoculation with *Fusarium sp, Pythium sp.* was reisolated from all the rhizomes that showed symptoms of rhizome rot. On combined inoculation studies the aggravation of the disease was noted in the treatment with *Pythium +Fusarium + Rhizoctonia.* The results of all the three series of inoculations *viz*., individual, sequential and combined inoculations clearly revealed that *Pythium* is the principal causal

organism of rhizome rot disease of turmeric. *Fusarium* and *Rhizoctonia* did not show any symptoms on its own thereby showing no role of these pathogens in disease initiation. They can only aggravate the disease as secondary colonizers.

4.2.2 Characterization of the pathogen (s) using morphological and molecular aids.

4.2.2.1 Morphological characterization of the pathogen

Eighty one isolates of *Pythium* collected from different turmeric growing tracts of South India were tested for their pathogenicity under greenhouse conditions. 45 isolates showed pathogenicity and were characterized morphologically under various conditions. 32 isolates were identified as *P. aphanidermatum* showing its role as a predominant pathogen in rhizome rot of turmeric. Eight isolates were identified as *P. myriotylum*.

4.2.2.1. a. Colony morphology and growth rate: variations were observed in growth rate as well as colony morphology among the *Pythium* isolates. Among the isolates, IISR TP 29 and IISR TP 10 showed highest growth rate on PDA.

4.2.2.1. b Virulence of the isolates: *Pythium* isolates showed variation in the virulence test. Among the isolates IISR TP 09, IISR TP 16, IISR TP 20, IISR TP 29, IISR TP 65 and IISR TP 71 were found to be most virulent isolates.

4.2.2.1. c Microscopic characterization: The isolates were tested for the production of reproductive structures oogonia and antheridia for characterization of the isolates. All the isolates were induced for the production of reproductive structures like sporangia, oogonia and antheridia using grass leaf method. The isolates of *P.aphanidermatum* produced toruloid sporangia. IISR TP 11, IISR TP 48, IISR TP 75, IISR TP 12 and IISR TP 66 did not sporulate by any of the methods tested.

4.2.2.2 Molecular characterization of *Pythium* isolates: PCR - RFLP analysis of *Pythium* sp. causing rhizome rot of turmeric

The combination of universal primers ITS1 and ITS4 generated a 900-bp fragment during PCR for all the isolates of *Pythium* used for the present study. The amplified products were digested with restriction enzymes *Msp* I, *Taq* I and Alu I for PCR-RFLP analysis. RFLP finger print data grouped the isolates into two main groups (major group I and II) with 10% similarity. The isolates TP17, TP42, TP34, TP15 and TP78 formed a sub cluster I with 100% similarity and this sub cluster showed 95% similarity with TP 16 and 85% with TP02. The isolate TP 79 showed a 39% similarity with sub cluster I. Second major group consisted of two main subgroups with clusters and sub clusters First subgroup consisted of one cluster with 60% similarity. Among these 28 isolates, 26 formed a sub cluster with 100% similarity. Second subgroup consisted of a total of nine isolates with 7 sub clusters. Among these isolates, TP75 and TP66 formed a group with 100% similarity. The isolates

TP 19, TP 52 formed a sub cluster with the isolates TP 45, TP 11 with 91 % similarity. The isolate TP69 grouped in a different sub cluster with 59% similarity. The isolates TP04 and TP12 showed 56% similarity and grouped separately. ITS-RFLP results indicated that variations among the isolates occur within and between different geographical locations. Based on genetic distances and cluster analysis, it could be found out that a greater level of intraspecific and interspecific variation was observed among all the isolates tested.

4.2.3 Study on the biology of the pathogen

The results were compared with the soil pH recorded during the survey and found that high disease incidence (DI) under natural conditions is correlated with pH above 6.0. The field with pH less than 6.0 showed low disease incidence.

4.2.3.2 Effect of pH and temperature on the germination of oospores

The germination of oospores of two isolates were pooled and analyzed. The oospore germination occurred over a temperature ranging from 15-35^{o}C. The germination of oospores was found higher at temperatures 25 and 35^{o}C. However, there was no significant difference with germination percentage at 15^{o}C. The percentage of germination was comparatively higher at pH ranging from 6.0-7.5. The *in vitro* study on the germination of oospore and growth of the mycelium showed that the favorable range was 6.0 - 7.5.

4.2.3.3 Study on the host range of the pathogen

Out of the six members tested to study the host range of the pathogen, *C. zeodaria* and *Z. officinale* developed symptoms of rhizome rot within seven days of inoculation. Variation in the symptoms was noted among these. *Z. officinale* was found more susceptible showing lesion on pseudo stem and yellowing of the leaves. The younger leaves turned yellow with in seven days.

4.2.3.4 Fungicide sensitivity of *Pythium*: Among the fungicides tested Metalaxyl-Mz was found to inhibit the pathogen at 200 ppm with EC 50 of 78.46. *P. aphanidermatum* showed highest sensitivity to Metalaxyl-Mz when compared to other fungicides. The information on the sensitivity of the pathogen towards these fungicides was used to undertake field trial in diseased area. EC 50 value of the fungicides showed significant differences. The minimum EC50 value was showed by Metalaxyl-Mz (78.46) followed by Copper Oxycholoride (87.02).

4.3. Study on disease management

4.3.1 Isolation and evaluation of *Trichoderma* from turmeric rhizosphere

Among the 22 isolates six isolates were obtained from Kerala, eight isolates from Tamil Nadu and four isolates each from Andhra Pradesh and Karnataka.

4.3.2 Evaluation of the biocontrol potential of *Trichoderma* isolates against *P. aphanidermatum* (*in vitro*)

4.3.2.1 Dual culture assay for preliminary detection of antimicrobial activity

Among the six isolates, four isolates viz. IISRCLT 102, IISRCLT 107, IISRCLT 110 and IISR CLT 114 showed higher scale (S1-100% overgrowth) with in three days. Out of twenty two isolates six isolates via, IISRCLT 102, IISRCLT 107, IISRCLT110, IISRCLT 114, IISR CLT118, and IISR CLT 121 showed mycelial inhibition of above 70% *in vitro.*

4.3.2.2 Effect of Non Volatile Metabolites: The non volatile metabolites produced by IISRCLT 102 (37.78%), IISRCLT 107(38.52), IISRCLT 114 (42.22%) and IISRCLT 110 (46.30%) were comparatively effective against the pathogen.

4.3.2.3 Effect of Volatile Metabolites: The highest inhibition by volatile metabolites was shown by IISRCLT 118 (84.82%) and IISRCLT 121(82.22%).

4.3.3 Screening of shortlisted *Trichoderma* isolates for their efficacy as biocontrol agent

4.3.3.1 Extracellular enzyme production: The isolates varied in the production of extracellular enzymes. IISRCLT 110 and IISRCLT 114 were found to produce all the three enzymes assayed.

4.3.3.2 Induction of plant defense enzymes: The isolates showed variation in their induction of plant defense enzymes. Leaves of turmeric plants treated with IISRCLT 102, IISRCLT 107, IISRCLT 110 and IISRCLT 114 showed high PAL and PRX activity on 5th day after inoculation where as no induction of these enzymes were found in root and rhizomes even on 15^{th} day after inoculation. The activity of the enzymes was found slightly lower on 15^{th} day than on 5^{th} day. PPO activity was not found increased in all the three parts on 15^{th} day for all the isolates.

4.3.3.3 Fungicide sensitivity of shortlisted *Trichoderma* isolates: None of the potential isolates were found to be Carbendazim resistant. All the isolates were sensitive to Captan-hexaconazole above 1000 ppm. Potassium phosphonate showed no sign of inhibition even @3000ppm for all the isolates. None of the other chemicals were found inhibitory at the recommended dosages except Cheshunt compound. These promising isolates were evaluated under pot culture conditions and are also being evaluated in the field for the disease management.

4.3.4.4 pH tolerance of shortlisted *Trichoderma* isolates: In the present study, all the six isolates showed no significant variation at pH ranging from 4.5-8.5. However, there is difference in growth rate during the initial 48 h of incubation. All the isolates showed an initial setback in growth at acidic (< 5.0) and alkaline pH (> 7.5).

4.3.4 Pot culture evaluation of the shortlisted *Trichoderma* isolates against *P. aphanidermatum*

4.3.4.1. Study on the rhizosphere colonization of shortlisted *Trichoderma* isolates

The rhizosphere colonization of the *Trichoderma* isolates is given in Table 30. Among the isolates tested IISR CLT 107 showed higher colonization of 5.06 log cfu/gm of the soil after 180 days which is at par with IISR CLT 110 (4.86 log cfu/gm).

4.3.4.2 Evaluation of shortlisted *Trichoderma* isolates against *P. aphanidermatum* by challenge inoculation

Under challenge inoculation, the disease incidence varied from 0-73.33% and among the treatments IISRCLT 107 and IISR CLT 110 showed least disease incidence (0.0%) when compared to control (73.33%). Similarly IISRCLT 107 showed higher survivability and colonization in rhizosphere soil after 180 days of inoculation whereas IISR CLT 118 showed least survivability and colonization.). IISR CLT 110 showed no disease incidence followed by IISR CLT 114 (6.67%) and IISRCLT 107 (13.33%) when compared to control (73.35%). The control plants took infection within 10 DAI with the pathogen.

4.3.4.2. a Disease Potential Index (DPI) estimation

Disease potential index (DPI) of *Pythium* in the inoculated pots showed a significant difference among the treatments (Fig 12). Inoculation with *Trichoderma* strains IISR CLT 107, IISR CLT 110 and IISR CLT 114 showed significant reduction in *Pythium* population. With IISR CLT 114, *Pythium* population was detected only up to 90th day and in IISR CLT 107 and IISR CLT 110 , the population was detected only up to 120th day.

4.3.5 Disease management trial with chemicals.

4.3.5.1 a. Estimation of total fungal and bacterial population: Field trial conducted in 2007-08 season showed significant differences with regard to rhizosphere fungi and bacteria among the treatments at 30 days intervals (Table 34). A decline in the fungal population was observed in all the treatments compared with initial population and control. However, no general trend was noticed among the treatments except in cheshunt compound where a sharp decline in the population was observed at 30 and 60 days after treatment.

4.3.5.1 b. Disease incidence and yield: Among the different fungicides tested, COC proved to be the most efficient with minimum DI of 8% followed by Metalaxyl-Mz (11.33%) during 2007-08. Considering the overall mean for the four field trials, Metalaxyl-Mz was found to be more efficient followed by COC. All the other fungicides which proved their efficacy against *P.aphanidermatum in vitro* showed no consistent results in the field.

The yield data also showed significant difference between control and fungicide treatments. COC showed the maximum yield followed by carbendazim and Metalaxyl-Mz during 2007-08 season. The highest increase in yield was observed in COC (4.43%) followed by Carbendazim (2.90%) and Metalaxyl-Mz (2.56%). Potassium phosphonate and carbendazim + mancozeb showed no significant increase in the yield. The lowest yield was recorded in treatments with carbendazim + mancozeb.

During 2008-09, COC showed highest yield (91.17 kg/plot) followed by Metalaxyl-Mz (88.83 kg/plot) in field I which was 32.13 and 28.74% increase respectively over control followed by Cheshunt compound (22.94%). The least increase was from potassium phosphonate (8.22%). Similar results were also noted in field II of the same season with the exception of captan hexaconazole, which recorded the least increase in yield (6.83%). Similarly, in 2009-2010 season, the highest increase was observed in plants treated with metalaxyl + mancozeb (11.66%) followed by COC (8.53%). No increase in yield was observed in potassium phosphonate and carbendazim + mancozeb. The mean yield across both the seasons revealed that Metalaxyl-Mz and COC treatments increased the yield and markedly reduced DI.

4.3.6.1.1 Disease management trial with biocontrol agents.

The germination count was taken to study the effect of the isolates on germination. As observed in the case of chemical trials, no significant difference was observed among the treatments in comparison with control in all the three trials.

4.3.6.1 a. Estimation of rhizosphere colonization of *Trichoderma* isolates: In the field I during 2008-09 season, the isolates showed no significant difference in their population. Among the isolates, IISRCLT 102, IISRCLT 107, IISRCLT 110 and IISRCLT 114 showed greater population up to 120th DAI whereas a gradual decrease in the population was noticed in IISRCLT 121 inoculated rhizosphere soil samples from 30th DAI. Similar result was observed in field II of the same season, where IISRCLT 107, IISRCLT 110 and IISRCLT 114 maintained greater population up to 120 DAI in comparison to other isolates. IISRCLT 110 had highest population level (3.030 log cfu/g) at 180th DAI in comparison with other isolates. Here also IISRCLT 118 and IISRCLT 121 showed least population level from the 30th DAI. These results were found to be consistent in third trial also.

4.3.6.1 b. Estimation of total fungal and bacterial population: Among the treatments, no profound change was observed in the fungal population up to 180 DAP.

4.3.6.1 c. Disease incidence and yield: The treatments showed significant difference in the rhizome yield in all the three trials conducted. The two isolates *viz*., IISR CLT 107 and IISR CLT 110 were found to be the most promising isolates based on the three field trials conducted. The isolates were effective in reducing the disease incidence and thereby increasing the yield of turmeric.

4.3.7 Integrated disease management

The fungicides and biocontrol agents which proved their efficacy against rhizome rot in the initial two years were evaluated as a part of integrated management strategy. The percentage of germination was showed no significant difference among the treatments.

4.3.7.1. a Estimation of rhizoshere colonization of *Trichoderma* isolates : The initial population of the biocontrol agents showed no significant difference in their population. However, the isolates showed a gradual decrease in the population at 60 DAI. The individual inoculation of the potential isolates showed high population level up to 150 DAI in comparison with those integrated with chemicals. No significant difference was noted among the isolates after 180 DAI.

4.3.7.1. b Total fungal and bacterial population in IDM

The total fungal population under various treatments showed significant differences for initial 180 DAI. The fungal populations under the integrated treatments were found to be at par with chemical alone after initial 30 days. The fungal population in the biocontrol treatment showed higher population level than other treatments. IISRCLT 110 and IISRCLT 107 treatments maintained high population level till 180 DAI.

4.3.7.1. c Disease incidence and yield: The data on DI showed significant differences among the treatments (Table 60). The lowest DI was observed in plants treated with Metalaxyl-Mz mancozeb (7.07%). This was followed by IISRCLT 110 alone (7.09 %) and Metalaxyl-Mz + IISRCLT 110 (7.22 %). The disease incidence was relatively low in all the treatments compared to control. The combination of Metalaxyl-Mz + IISRCLT 107 (8.46%) also showed low DI which was at par with Metalaxyl-Mz + IISRCLT 110 and IISRCLT 110 alone.

5. Summary and Conclusion

- Of the 37 different locations in 12 districts of southern states of India surveyed, the highest disease incidence (32%) was recorded from Vallabhapuram in Guntur dist. of Andhra Pradesh whereas Cheraooru in A.P and Vellarikunnu in Kerala recorded the least (0%) incidence.
- A total of 118 rhizome rot diseased samples collected, *Pythium* spp. dominated in majority of the diseased samples (72.88%) followed by *Rhizoctonia spp.* (30.50%) and *Fusarium* spp. (27.12 %).
- Pathogenicity studies with individual inoculations of *Pythium* sp, *Rhizoctonia* and *Fusarium* sp. revealed that *Pythium* takes infection in 10 days of inoculation where as *Fusarium* sp. or *Rhizoctonia* sp. did not show any symptoms even after 90 days of inoculation.

- Results of individual, sequential and combined inoculation of *Pythium*, *Fusarium* and *Rhizoctonia* showed that *Pythium* is the primary causal organism and the other two are secondary colonizers. *Fusarium* is found to cause aggravation of the disease when inoculated in a sequential manner as described.

- Among the *Pythium* species isolated, *P. aphanidermatum* was found to be the predominant pathogen. Apart from it, *P. myriotylum* and other five non sporulating isolates were also found in association with the disease. Morphological characterization of these isolates revealed that out of 45 pathogenic *Pythium* isolates, 32 were identified as *P.aphanidermatum*, 8 as *P. myriotylum* and 5 other non sporulating isolates. The *Pythium* isolates showed variations in their colony morphology, growth rate and virulence.

- Molecular characterization of 45 isolates of *Pythium* using restriction enzymes viz. *Taq* I, *Msp* I and *Alu* I revealed both intra and inter specific variability in their mRNA ITS region. The dendrogram of ITS RFLP grouped these isolates into two major groups and a sub group. Majority of *P. aphanidermatum* and *P. myriotylum* were included in this main and sub cluster respectively.

- The study on oospore germination revealed that the soil with slightly acidic pH or towards neutral or slightly alkaline pH favours the growth of the pathogen. The percentage of germination was comparatively higher at pH ranging from 6.0-7.5.

- The host range study of *P. aphanidermatum* on six Zingiberaceae members revealed that *Z. officinale*, *C. zeodaria* and *C. aromatica* can act as host for the pathogen and the practice of crop rotation with these crops may cause heavy loss in turmeric cultivation. Hence rotation of these crops with turmeric may be avoided as a part of disease management strategy.

- Fungicide sensitivity study *of P. aphanidermatum* showed Metalaxyl-Mz as the most effective fungicide followed by Copper oxychloride with minimum EC 50 value.

Turmeric being an export oriented crop, disease management practices which provides pesticide free product would be more receptive. Since biocontrol form an active component of disease management (mostly under IDM) which is more ecofriendly and economical and *Trichoderma* species form the most popular and effective candidate, much effort has been made to isolate potential biocontrol strains of *Trichoderma* that can combat the disease individually and in integrated approaches.

- Preliminary screening of 22 isolates of *Trichoderma* collected from different geographical region showed variations in their antagonistic activity against *P.aphanidermatum in vitro*. Among the isolates tested by dual culture, six isolates *viz*., IISR CLT 102, IISR CLT 107, IISR CLT 110, IISR CLT 114, IISR CLT 118 and IISR CLT 121 showed inhibition of over 70%. Some of these isolates induced hyphal evacuation and cytoplasmic coagulation of the mycelium of pathogen. Study on the effect of non volatile metabolites revealed that the

isolates IISR CLT 103, IISR CLT 107 and IISR CLT 118 are highly effective against the pathogen. IISR CLT 118 and IISR CLT 121 showed highest volatile activity. Based on these results six isolates viz. IISR CLT 102, IISR CLT 107, IISR CLT 110, IISR CLT 114, IISR CLT 118 and IISR CLT 121 were shortlisted for further studies.

- Out of six short listed isolates, IISR CLT 102, IISR CLT 110 and IISR CLT 114 produced all the three enzymes tested *viz*., amylase, cellulase and pectinase. IISR CLT 118 and IISR CLT 121 were not found to produce cellulase. Pectinase enzymes were found to be produced only by IISR CLT 102, IISR CLT 107, IISR CLT 110 and IISR CLT 114.

- All the six isolates were found to induce the production of defense enzymes in leaf of turmeric plants. The isolates showed variations in the induction of plant defense enzymes in different parts of the plant viz. root and rhizome. Among the isolates IISR CLT 107 was found promising by inducing PO, PPO and PAL in the leaves of inoculated plants.

- In fungicide sensitivity tests, IISR CLT 110 was found least sensitive to copper oxychloride and Metalaxyl-Mz showing its compatibility with these fungicides. The potential of this isolate offers its exploitation in integrated disease management of rhizome rot.

- All the shortlisted isolates showed wide adaptability towards different ranges of pH. This property can be exploited for its wide application in different turmeric growing tracts.

- The pot culture studies showed IISR CLT 107, IISR CLT 110 and IISR CLT 114 as promising isolates to reduce the inoculum load of the pathogen. The higher colonization capacity of these isolates also proves their ability to sustain in the soil throughout the crop season thereby giving protection against the pathogen.

- The field trial conducted during 2007-10 in disease prone area of Annur showed that Metalaxyl-Mz @ 0.125% and Copper oxychloride @ 2.0% are effective in managing the disease. **Thus dipping seed rhizomes for 30 min in either of the fungicides viz. Metalaxyl-Mz @ 0.125% or Copper oxychloride @ 2.0% followed by subsequent drenching of the same at the time of planting and repeated applications thrice at 30 days interval can form a disease management package**. The use of these fungicides alone or alternating with each other can reduce the disease incidence and also reduce the chance of developing fungicide resistance in pathogen. The study also highlights the importance of seed treatment before planting.

- The biocontrol trial using different *Trichoderma* isolates revealed IISR CLT 107 and IISR CLT 110 as most promising isolates to manage the disease. These isolates were identified as ***Trichoderma harzianum*** **Rifai (NFFCI 2092) and *T. longibrachiatum* Rifai (NFFCI 2093)** respectively. **The practice of planting rhizomes after priming with *Trichoderma* spore suspension at 1 kg rhizome L^{-1} containing a spore load of $1x10^8$ cfu ml^{-1} for 30**

min with 5% starch solution and its repeated soil application at 30days intervals was found effective in managing the disease. These isolates were found as effective as chemical treatment when used individually. The use of these isolates as seed treatment and also as soil application at the earlier stage of the crop will improve the biocontrol efficacy. It is also found that the total bacterial or fungal population is unaffected by soil application of these isolates.

- The integrated management trial showed that a combination of Metalaxyl - Mz + IISRCLT 110 is highly promising in managing the disease. Hence it can be concluded **that the Seed dressing and soil drenching with Metalaxyl-Mz 0.125% at the initial stage of the crop in combination with IISR CLT 110 @ 5 lit/block (average 150plants/block) having a cfu of 10^8 ml $^{-1}$ at the time of planting and at 30 day interval thrice will greatly help in reducing the disease incidence and increasing the yield and this combination will help to minimize the use of chemicals.**

The main objectives of present study were confirming the etiology of rhizome rot of turmeric and to develop a management strategy for the same. From the results of the present study, it is confirmed that

- ***Pythium aphanidermatum* (Edson) Fitz. is the predominant pathogen in causing the disease**.
- Isolated and identified two potential *Trichoderma* isolates viz. ***Trichoderma harzianum* Rifai and *T. longibrachiatum* Rifai** for the biological control of rhizome rot**.**

Based on the actual causal organism, the following packages are found suitable to combat the disease

- **Dipping seed rhizomes for 30 min in either of the fungicides viz. Metalaxyl-Mz @ 0.125% or Copper oxychloride @ 2.0% followed by subsequent drenching of the same at the time of planting and repeated applications thrice at 30 days interval.**
- **The practice of planting rhizomes after priming with *Trichoderma* spore suspension at 1 kg rhizome L^{-1} containing a spore load of 1x10^{8}cfu ml^{-1} for 30 min with 5% starch solution and its repeated soil application at 30days intervals.**
- **Seed dressing and soil drenching with Metalaxyl-Mz 0.125% at the initial stage of the crop in combination with IISR CLT 110 @ 5 lit/block (average 150plants/block) having a cfu of 10^8 ml $^{-1}$ at the time of planting at 30 day interval thrice.**

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Anoop, K. and Suseela Bhai, R., 2009. Evaluation of Potential *Trichoderma* isolates for the management of rhizome rot disease in Turmeric (*Curcuma longa* L.) Ab: 5th International national Conference on Phytopathology, New Delhi, Nov 10-13, 2009.

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